

# OSTEOBLAST DIFFERENTIATION AND EXTRACELLULAR MATRIX MINERALIZATION IN RESPONSE TO PORE SIZE AND SHAPE: STATIC CULTURE

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## ABSTRACT

Poly(ester urethane)urea (PEUUR) foams have been designed to cure in situ upon injection to form porous scaffolds. In this study, the effects of water concentration and polyester triol composition on the physicochemical, mechanical, and biological properties of PEUUR foams were investigated. A liquid resin (lysine diisocyanate: LDI) and hardener (poly( $\epsilon$ -caprolactone-co-glycolide-co-DL-lactide) triol, tertiary amine catalyst, anionic stabilizer, and fatty acid-derived pore opener) were mixed, and the resulting reactive liquid mixture injected into a mold to harden. By varying the water content over the range of 0.5 – 2.75 parts per hundred parts polyol, materials with porosities ranging from 89.1 – 95.8 vol-% were prepared. Cells permeated the PEUUR foams after 21 days post-seeding, implying that the pores are open and interconnected. In vitro the materials yielded non-cytotoxic decomposition products, and differences in the half-life of the polyester triol component translated to differences in the PEUUR foam degradation rates. We anticipate that the injectable PEUUR foams will present compelling opportunities for bone tissue engineered and delivery systems due to their favorable biological and physical properties.

## 1. INTRODUCTION

Porous poly(esterurethane urea) (PEUUR) scaffolds have been reported to degrade to non-toxic by-products and support the migration of cells and ingrowth of new tissue (Zhang et al., 2000; Guelcher et al., 2006). These materials can be cast into objects having a desired shape by reactive liquid molding of two components. In a previously reported study, we synthesized two-component PEUUR foams by a one shot reactive liquid molding process without the intermediate

prepolymer synthesis step. An LDI resin was contacted with a hardener comprising a polyester triol, water, triethylene diamine (TEDA) catalyst, sulfated castor oil and polyethersiloxane stabilizers, and a calcium stearate pore opener (Guelcher et al., 2006). The objective of the study is to address if pore size and shape of poly(ester urethane)urea (PEUUR) polymers influence osteoblast cell differentiation and upregulate matrix mineralization under static cell culture condition.

## 2. MATERIALS AND METHODS

PEUUR foams were prepared by reactive liquid molding of two components: (a) lysine methyl ester diisocyanate (LDI), and (b) a hardener comprising polyester polyol, water, catalyst, stabilizer, and pore opener. Three 900-Da poly( $\epsilon$ -caprolactone-co-lactide) triol compositions with half-lives ranging from 30 – 230 days were synthesized from a glycol starter using published techniques. Six foams were used with water content in the hardener that varied from 0.5 – 2.75 parts per hundred parts polyol (pphp). These foams were labeled A-F. Water reacts with LDI to form gaseous carbon dioxide, which functions as a blowing agent. The reactions of LDI with water and polyol were catalyzed by 3 pphp triethylenediamine. The height of the rising foam was measured versus time and shrinkage was calculated as the reduction in foam volume after 24 h. MedPor and Vitoss were used as controls for this experiment.

### 2. 1 Scanning Electron Microscopy (SEM)

SEM was performed to determine the morphology of pores, cell attachment on the polymer surface, and cell migration into the pores. Statically seeded cells on PEUUR foams were fixed on the foams in 2.5%glutaraldehyde and postfixed in 1% Osmium tetroxide in 0.1M sodium cacodylate buffer (pH7.2). Foams were then dehydrated in ascending

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grades of ethanol (50%-100%) and critical point dried. Samples were bisected sagittally to expose the inner core of the foam, attached to an SEM stub with double-sided tape, and sputter coated with gold at three different angles, using a Pelco SC-6 Sputter Coater (Redding, CA). Samples were then examined with a Hitachi 2460N Scanning Electron Microscope (Tokyo, Japan). Digital images were obtained using Quartz PCLimage management software.

## 2.2 Fourier Transformation-Infrared Spectroscopy (FT-IR)

The compositions of the materials and degradation products were assessed by micro FT-IR. 100 $\mu$ l of the media containing polymer degradation products was collected and vacuum dried in an Eppendorf Vacuofuge™ (Hamburg, Germany) overnight and the powdered samples were directly analyzed under the FT-IR microscope. Spectra were collected in the reflectance mode with a resolution of 4 cm<sup>-1</sup> and 150 scans. Peak analysis was analyzed using the software.

## 2.3 Cell Culture System And Assays

Three cell lines were used to evaluate cell-material response, (a) MC3T3E1, (b) MG63, and (c) HMSC. Briefly, 5 $\times$ 10<sup>4</sup> cells were statically seeded onto each PEUUR scaffold, and cultured in a 24 well tissue culture plates for time points of 1, 7, 14, 28 days. The MC3T3's were cultured in  $\alpha$ -Minimum Essential Medium (MEM), MG63 in advanced Dulbecco MEM and hMSC in mesenchymal stem cell basal medium (MSCBM). For osteoblast differentiation and mineralization study, MC3T3 cells were treated with  $\alpha$ -MEM with 50 mg/mL of ascorbic acid, 10mM of  $\beta$ -glycerophosphate and 1mM dexamethasone.

## 2.4 Osteoblast Differentiation And Mineralization Markers

For cell-ingrowth, the foams seeded with cells were fixed in 10% neutral buffered formalin and dehydrated in ascending grades of alcohol, embedded in paraffin wax and 5 $\mu$ m thin sections were taken and stained with hematoxylin and eosin. Immunofluorescent staining was done on the histology sections for osteoblast differentiation

markers Osx (Osterix), Type-I Collagen and Runx-2. Osteoblast mineralization was determined using 8 $\mu$ g/ml of Tetracycline HCl added to 28 days cell culture, 2 days prior to the harvest and observed under fluorescence microscope. FT-IR was employed to assess the functional groups of the polymers before and after seeding cells.

# 3. RESULTS AND DISCUSSION

## 3.1 Density, porosity, and pore morphology

SEM images of the six foams are shown in Figure 1. The pores are irregularly shaped and vary in size from about 100–1000  $\mu$ m. The pores in the P7C2G1LW200 material were generally larger, with some pores >1 mm. The densities and porosities of the PEUUR foams are listed in Table 3 and range from 60 – 130 kg m<sup>-3</sup> (89 – 96 vol-% porosity). As expected, the density of the foams decreased and the porosity increased with increasing water concentration. However, at water concentrations greater than 2.75 pphp, the foams were unstable and contained voids (>5 mm). Considering that the lowest attainable density for stable, void-free foams was 60 kg m<sup>-3</sup> (2.75 pphp water), we suggest that 60 kg m<sup>-3</sup> represents the lower density limit for useful materials using water as the only blowing agent.

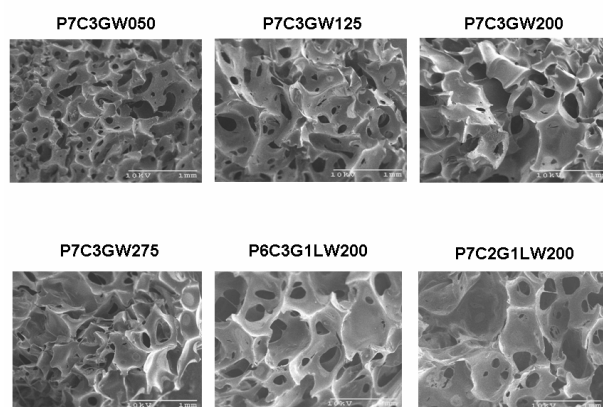


Figure 1. SEM of Polyurethane foams showing different pore size.

## 3.2 Infrared spectroscopy

IR spectra for all six materials are presented in Figure 2. The absence of an NCO peak at 2285 – 2250 cm<sup>-1</sup> implies that there is a negligible amount of free NCO (Kothandaraman et al., 1994; Socrates 1994). Note that the peak at 2353 cm<sup>-1</sup> varies for

each sample. This peak is assigned to Poly(ester urethane)urea scaffolds: effects of water and polyol composition the C=O stretching vibration of carbon dioxide and the variation between materials is presumed to stem from differences in background subtraction (Pecsok and Shields 1968). Characteristic stretching and deformation vibrations are observed for urethane, urea, and ester groups, as shown in Figure 2.

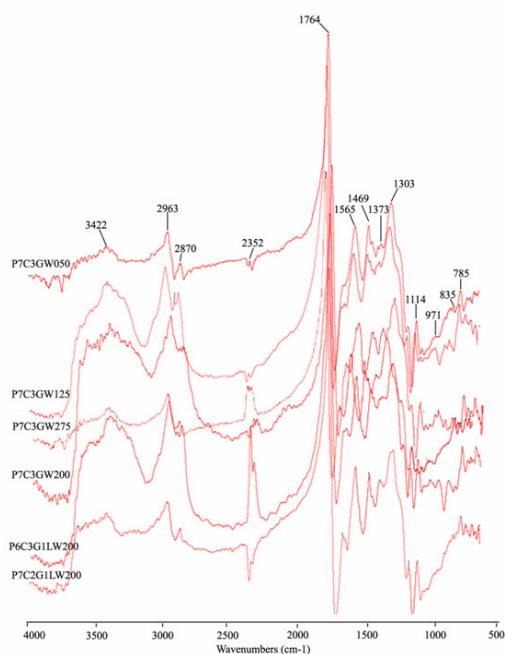


Figure 2. FT-IR spectra of PEUUR foams

The peak at  $1765\text{ cm}^{-1}$  corresponds to ester and urethane C=O stretching vibrations and is consistent with that previously reported ( $1723\text{ cm}^{-1}$ ) for the adduct of glucose and LDI (Zhang et al., 2002). The IR data imply that the materials have completely cured after 24 h at room temperature.

SEM images of the statically seeded foam surface revealed good cell attachment and anchorage both on the surface and within the pores of the foams after 28 days. More than 80% of cells exhibited flat cell morphology on the surface and populated the pores of the polymer (Figure 3). Due to the higher porosity of the polyurethane foams (90 – 95%) relative to the Medpor material (29%), the polyurethane foams have a higher specific surface area, which is conjectured to contribute to the increased number of cells attached to the foams. Differences in the morphology and interconnectivity of the pores, as well as the

chemical composition of the materials, could also promote differences in cell attachment.

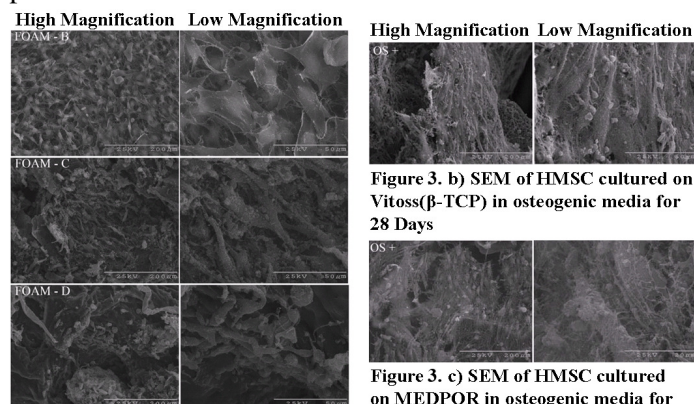


Figure 3. a) SEM of HMSC cultured on PEUR foams in osteogenic media for 28 days

Figure 3. b) SEM of HMSC cultured on Vitoss(β-TCP) in osteogenic media for 28 days

Figure 3. c) SEM of HMSC cultured on MEDPOR in osteogenic media for 28 days

Further, H & E staining of the prepared foams showed robust cell infiltration into the PEUR foams suggesting the pore size and interconnectivity support cell penetration and migration (Figure 4.)

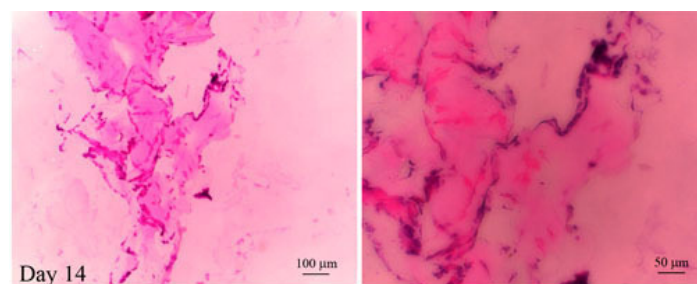


Figure 4. Hematoxylin and Eosin stained sections of MG63 osteoblast cells seeded on PEUR polymer foam after 14 days.

HMSC, MG63 and MC3T3 cells differentiated into mature osteoblast cells as evident from the immunostaining for osteoblast differentiation markers Runx-2, Osx and Type-I collagen (Figure 5). Matrix mineralization by tetracycline labeling (Figure 6) showed increased mineralization after 28 days.

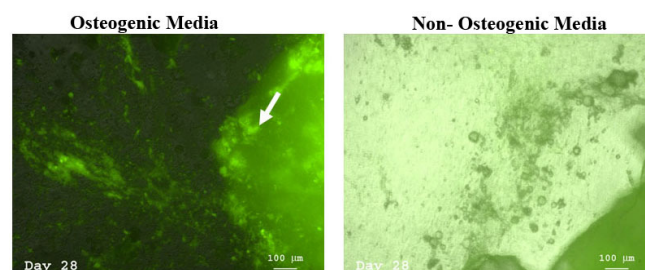


Figure 6. Tetracycline labeling show (white arrow) mineralized matrix within the PEUR foams in osteogenic media

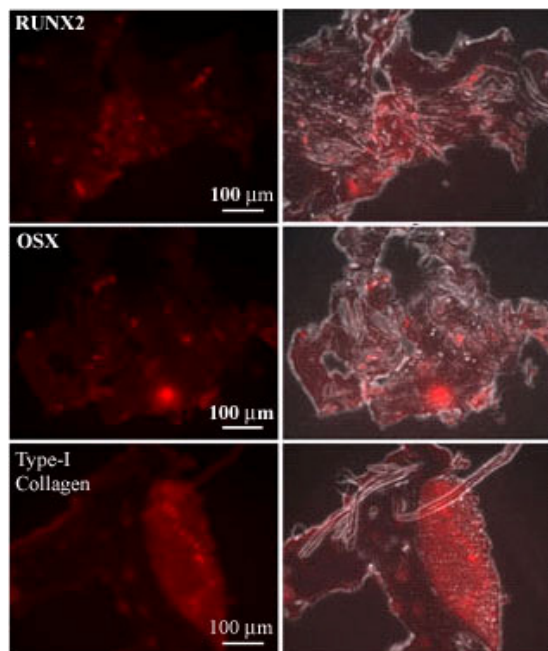


Figure 5. Immunofluorescence staining for osteoblast mineralization markers on MG63 osteoblast cells seeded on PEUR foam after 14 Days

#### 4. CONCLUSION

Polyurethane foam scaffolds were prepared by casting reactive liquid mixtures in open molds. The foams rise and gel in about 20 minutes with minimal temperature increase, and consequently are potentially injectable. Porosity and pore size can be tuned to targeted values by varying the composition of the hardener. All the foams supported cell attachment. Significantly, cells cultured on the foams supported osteoblast differentiation and matrix mineralization. Therefore, PEUR polymers have potential application as injectable delivery systems that would be useful in bone tissue engineering applications.

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